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# THE ROLE OF CALCIUM IN THE SECRETORY RESPONSE OF THE ADRENAL MEDULLA TO ACETYLCHOLINE

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It is commonly believed that acetylcholine (ACh) is the physiological transmitter of sympathetic nerve impulses at the adrenal medulla. The reasons are the following: the sympathetic nerve fibres that innervate the adrenal medullary cells are developmental homologues of the preganglionic fibres elsewhere in the sympathetic nervous system which are known to be cholinergic; on stimulation of the adrenal nerves an acetylcholine-like substance is released from the adrenal gland (Feldberg, Minz & Tsudzimura, 1934); ACh is a powerful stimulant of adrenal medullary secretion; and finally, transmission at the adrenal medulla is affected by a variety of drugs in much the same way as these drugs affect transmission at the established sites of cholinergic transmission at sympathetic ganglionic synapses.

The purpose of the present experiments was to study the mechanism by which ACh brings about adrenal medullary secretion and, as a first approach, to observe how the response of the gland to ACh might be influenced by changes in the ionic composition of the extracellular environment. Our experiments show that the excitant action of ACh on the adrenal medulla is dependent on the presence of calcium, and suggest that ACh evokes adrenal medullary secretion by causing calcium ions to penetrate the adrenal medullary cells.

Short accounts of these experiments were presented at the Oxford meeting of the Physiological Society and at the First International Pharmacological Meeting earlier this year (Douglas & Robin, 1961*a*, *b*).

## METHODS

All experiments were done on cats anaesthetized with chloralose (90 mg/kg I.V.) following ethyl chloride and ether. A tracheal cannula was inserted and artificial ventilation begun with minute volume sufficient just to suppress spontaneous respiratory movements. This was done to prevent any adrenal discharge which would otherwise have resulted from failure of ventilation during the later operative procedures which led to pneumothorax. A mid line abdominal incision was made from the xiphisternum to the pubis and the entire gastro-intestinal tract removed. The xiphisternum and lower ribs were retracted headwards to provide free access to the adrenal glands. One or both adrenal glands were then prepared

for perfusion. In some experiments one or both glands were perfused *in situ*; more often, however, one or other adrenal gland was removed from the animal and perfused *in vitro*.

*Perfusion of the adrenal glands in situ.* In a number of preliminary anatomical studies casts were made of the arterial supply to the adrenal glands by injecting thin liquid plaster of Paris under considerable pressure through a cannula in the abdominal aorta. By this means it was shown that the arterial supply was both complex and variable. Arterial twigs to the adrenal gland were seen coming from: (1) the adrenolumbar arteries and the phrenic artery, (2) the coeliac axis and superior mesenteric artery at various distances from their origins from the aorta, (3) the renal arteries at varying positions between the abdominal aorta and kidneys, and (4) the abdominal aorta (especially its ventral aspect) at varying distances caudal from the adrenals as far as, or even below, the origin of the renal arteries. Gross variation from animal to animal was apparent and it was obvious that any *close* arterial perfusion was impracticable. A more remote perfusion technique was therefore adopted as follows:

(1) A series of ligatures was placed around the border of the right kidney, and the renal vein and artery tied off close to the hilus.

(2) The right kidney was elevated and the adrenolumbar artery and vein tied off just lateral to the right adrenal gland.

(3) The diaphragmatic branches of the right adrenolumbar and coeliac arteries were tied along with any associated veins (the coeliac axis and superior mesenteric arteries had been tied and cut during evisceration). Neighbouring vascular shunts were occluded by cutting the right crus of the diaphragm between ligatures.

(4) The right greater and lesser splanchnic nerves were transected.

(5) While the right kidney was reflected medially (but not so as to interfere with the circulation of the right adrenal), ligatures were placed around the three or more pairs of perforating lumbar arteries which arose from the abdominal aorta on its dorsal aspect in the region to be perfused.

(6) The dorsal aspect of the aorta was completely freed from the prevertebral tissue to interrupt sympathetic nerve fibres that connect the adrenals and the sympathetic chain in this region (Vogt, 1952).

(7) Steps 1–4 were repeated on the left side.

(8) The aorta was tied immediately above the coeliac axis and perfusion begun through a glass cannula inserted into it about 1·5 cm below the renal artery and pointing headwards; this cannula was fitted with a bubble trap.

(9) The inferior vena cava was tied below the liver and above the entry of the right adrenolumbar vein. Perfusate was collected through a polythene cannula inserted into the vena cava at the same level as the arterial cannula.

(10) A third cannula, made of polythene tubing and fitted with a tap, was inserted into the coeliac axis. During the change over from one perfusion solution to another, fresh fluid was first washed vigorously through a side arm in the 'inflow' arterial cannula in the aorta, and then the tap of this third 'outflow' cannula was opened and the remaining dead space in the abdominal aorta washed out. In this way the effective dead space in the system was equivalent to the small volume of fluid in the fine arterial twigs supplying the gland.

(11) As soon as perfusion was begun the animal was killed by cutting open the thoracic aorta so that arterial pressure fell to zero and no blood could thereafter find its way into the perfused region through any untied anastomosis.

The gland was perfused at a constant pressure of about 70 mm Hg provided by a large reservoir of compressed air. Flow through the perfused region was about 3–4 ml./min. The total blood flow through both the cat's adrenals has been estimated to be approximately 1–3 ml./min (Stewart & Rogoff, 1917).

*Perfusion of the isolated adrenal gland.* During the course of the investigation it was found

more convenient for many purposes to isolate a single gland, either the right or the left, and perfuse it in the reverse direction through the adrenolumbar vein. This procedure had the advantage of limiting the perfusion to the adrenal tissue and thereby allowing a higher concentration of catecholamines in the perfusate. This was of especial value when, for example, the perfusate contained excessive amounts of potassium, which would tend, in large volume, to interfere with the bioassay. The preparation had the additional advantage of being comparatively simple to set up.

The procedure was as follows: The animal was eviscerated as previously described, and one or other kidney retracted to reveal the corresponding adrenolumbar vein. Any tributaries from extra-adrenal sources which joined this vein in its course across the gland to the inferior vena cava were tied and a fine polythene cannula connected to the perfusion system was tied into the vein lateral to the gland and pointing toward it. The adrenolumbar vein was then tied close to the vena cava and retrograde perfusion begun with a pressure of about 70 mm Hg. The thoracic aorta was then cut open to reduce systemic blood pressure to zero, and the adrenal gland cut free from the animal as rapidly as possible so that it would not be stimulated by discharges in the sympathetic nerves set up by the terminal asphyxia. The gland, attached to the end of the perfusion cannula, was transferred to a filter funnel filled with liquid paraffin. The effluent from the cut ends of the many small arterial twigs accumulated in the stem of the funnel and could be drawn off at appropriate intervals.

*Perfusion fluids.* Perfusion was usually carried out with phosphate-buffered Locke's solution of the following composition (mM): NaCl, 154; KCl, 5·6; $CaCl_2$, 2·2; $Na_2HPO_4$, 2·15; $NaH_2PO_4$, 0·85; glucose 10. When equilibrated with pure oxygen this solution had a pH close to 7·0. Similar solutions were used deficient in sodium, potassium or calcium. When sodium was removed the osmotic pressure of the solution was restored with sucrose (0·9 % NaCl was considered to be isosmotic with 9·25 % sucrose). Some solutions contained 5 or 10 times the normal amount of potassium (28 or 56 mM). In these solutions sodium was reduced by a corresponding amount. In some experiments to test the effect of various calcium concentrations bicarbonate-buffered Locke's solution of the following composition was used (mM): NaCl, 154; KCl, 5·6; $CaCl_2$, 0·5, 2·2, 8·8 or 17·6; $NaHCO_3$, 6; glucose 10. These solutions were equilibrated with 5 % $CO_2$ in $O_2$ and had a pH close to 7·0. In some experiments the perfusion fluid was pure sucrose in a strength (9·75 g/100 ml.) isosmotic with Locke's solution. Sometimes calcium (2·2 mM) was added to this sucrose solution. ACh chloride was added to any of these solutions when necessary in a concentration of $10^{-5}$ g/ml. All solutions contained ascorbic acid $10^{-5}$ g/ml. to inhibit oxidation of catecholamines. Perfusion was carried out at room temperature (24–26° C). Samples of perfusate were collected into flasks kept on ice, and then immediately frozen for subsequent assay.

It should be noted that we have observed no consistent or significant effect of ACh on the flow of perfusate through the adrenal. In some experiments the flow in the presence of ACh was identical to that in its absence: in other experiments the flow during perfusion with ACh was slightly above or below the levels found in ACh-free Locke's solution, but the effect was no greater than that which tended to occur spontaneously and which had no detectable influence on catecholamine output.

*Assay of catecholamines.* In response to electrical stimulation of the splanchnic nerves, or to ACh or potassium, the adrenal glands release the two catecholamines noradrenaline and adrenaline. The relative amounts vary widely, not only in different experimental conditions, but in apparently similar experimental conditions in different investigations (von Euler, 1956). Since our immediate interest was the total catecholamine output, the test object chosen for assay was the rabbit's thoracic aorta, which according to Furchgott & Bhadrakom (1953) is only very slightly more sensitive to noradrenaline than to adrenaline. The preparation was made as described by Furchgott (1960), and suspended at 37° C in Krebs's solution of the following composition (mM): NaCl, 118; KCl, 4·73; CaCl, 2·54; $KH_2PO_4$, 1·18; $NaHCO_3$, 24·9; glucose 11·09. This was equilibrated with 5 % $CO_2$ in oxygen and contained

the disodium salt of EDTA (ethylenediaminetetra-acetic acid) ($10^{-5}$ g/ml.) to chelate heavy-metal ions and slow oxidation of the catecholamines (Furchgott, 1960). Such a preparation almost always gave satisfactory contractions when 0·02–0·03 $\mu$g of adrenaline was added to the bath (8 ml.). A number of preparations were about ten times as sensitive. There is ample evidence that the smooth-muscle-stimulating substances released from the adrenal gland by splanchnic stimulation, ACh or potassium are adrenaline and noradrenaline. The active material in perfusates obtained in the present experiments caused contractions identical with those caused by adrenaline and was blocked to the same extent as adrenaline by partially blocking doses of the blocking drug phentolamine (Regitine). Assays were usually performed with doses equivalent to 0·05 $\mu$g or less of adrenaline (the values were expressed in terms of adrenaline bitartrate). At such levels discrimination between different doses was good and recovery prompt, so that tests could be made at intervals of a few minutes. It was found that by careful and repeated bracketing of test and standard samples an estimate of the adrenaline content of the 'test' sample could be expected not to deviate by more than about 10 % from the true value.

*Recording of contractions.* Shortening of the aortic strip was recorded by an isotonic lever with a load on the tissue of 4 g. The small doses of catecholamines used caused an extremely small shortening of the aortic strip (about 1 % of its length) and, with the 4 cm length we employed, required a friction-free and sensitive recording apparatus. Movement of the lever was therefore measured, amplified and recorded electrically as follows: A metal rod was suspended from the lever in such a way as to act as a moving core of a coil carrying alternating current. Changes in the position of the lever thus caused changes in the inductance of the coil. By incorporating this coil in a bridge circuit 'out of balance' currents were obtained which were amplified and used to drive an ink recorder. The over-all 'mechanical plus electrical' gain of the system was usually chosen to be about $\times 50$.

## RESULTS

### *Effect of ACh in Locke's solution*

Some preliminary experiments were carried out to establish appropriate conditions for stimulation by ACh. It was found that during perfusion with Locke's solution there was always some spontaneous output of catecholamines. The amount varied in different experiments, but was most commonly about 0·05–0·1 $\mu$g/min/gland (at the beginning of perfusion, the value was occasionally several times higher). Switching from Locke's solution to Locke's solution plus ACh $10^{-5}$ g/ml. always caused a large increase in catecholamine secretion. In the first 2 min the rate not uncommonly rose to 15 $\mu$g/min/gland; thereafter, as perfusion with ACh was continued the output fell to reach about 10 % of this rate by about the tenth minute (Fig. 1). The reasons for this falling output have not been explored. For the present purposes it was found satisfactory to make tests of the adrenal response to ACh lasting 30 sec or 4 min, and to repeat these tests at intervals of about 20 min. Although there was some fall off in the amount of catecholamines released in successive tests, the responses were maintained sufficiently well to permit experiments of the sort we performed (viz. Fig. 4).

The first group of experiments was designed to examine the effect of a

reduction in the extracellular concentration of one or other of the cations sodium, potassium, or calcium upon the catecholamine-releasing effect of ACh. The procedure adopted in these experiments is typified by the following experiment with K-free Locke's solution.

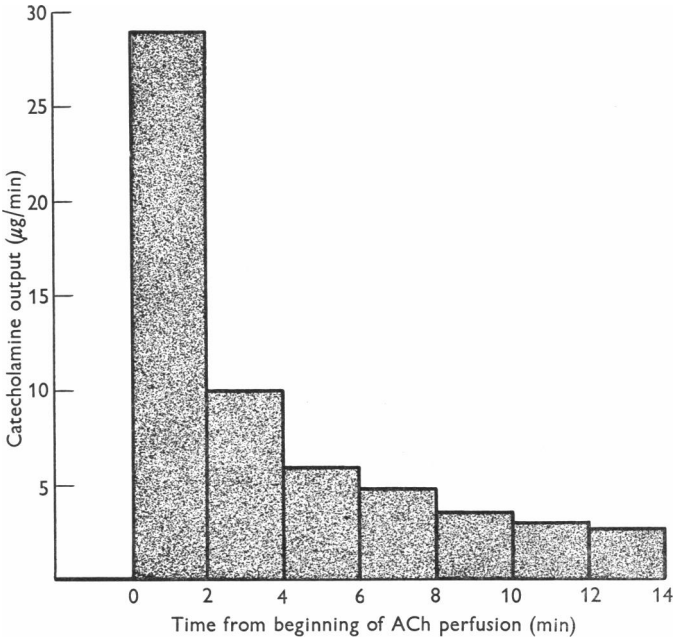


Fig. 1. The time course of catecholamine release during continuous perfusion of both adrenal glands with ACh. After a control period of perfusion with Locke's solution, during which catecholamine output was 0·064 μg/min (indicated by the thickening of the base line) perfusion was changed to Locke's solution containing ACh $10^{-5}$ g/ml. and successive 2 min samples taken.

*Effect of ACh in K-free Locke's solution*

In the experiment of Fig. 2*a*, an adrenal gland was isolated from the body and perfused *in vitro* with Locke's solution. After 16 min, during which four 'resting' samples of perfusate were collected for assay, perfusion was switched rapidly to a second solution identical with the first except that it contained ACh in a concentration of $10^{-5}$ g/ml. Perfusion with this ACh-containing medium was continued for 4 min and the perfusate was again collected for assay. Immediately following collection of this sample perfusion was changed to K-free Locke's solution. At the end of a further 16 min and collection of four more 'resting' samples, perfusion was again switched, this time to K-free Locke's solution containing ACh ($10^{-5}$ g/ml.) and another 4 min sample obtained. Then the whole sequence was repeated several times to observe further the stimulant effect of ACh in Locke's

solution and in K-free Locke's solution. All the samples were assayed and the output of catecholamines per minute during each collection period was calculated. As may be seen from Fig. 2*a*, which shows a typical series of responses obtained in this way, perfusion with ACh resulted in a huge outpouring of catecholamines amounting to several micrograms per minute. The absence of potassium from the perfusion fluid did not diminish the excitant effect of ACh on catecholamine release: on the contrary, in each of a number of tests it enhanced the effect.

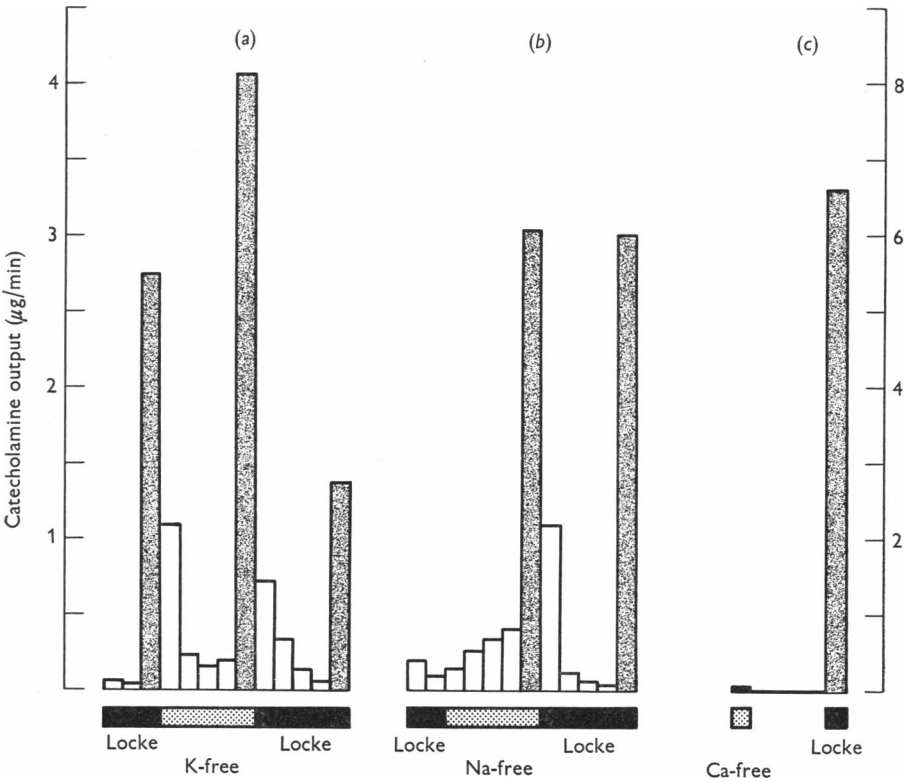


Fig. 2. The effect of omission of various cations on the amount of catecholamine released from the adrenal gland by ACh. The three experiments (*a*), (*b*) and (*c*) were performed on different adrenal glands perfused through the adrenolumbar vein with Locke's solution free from KCl (*a*), NaCl (*b*) or $CaCl_2$ (*c*). The deficient solutions are indicated by the stippled horizontal bars. Catecholamine output was measured in successive 4 min collection periods in each experiment. The solid vertical bars show the output in response to the addition of ACh $10^{-5}$ g/ml. during a 4 min period. The open vertical bars show the 'spontaneous' outputs of catecholamines. The scale on the left applies to (*a*): the scale on the right applies to (*b*) and (*c*). Since changing from Ca-free Locke's solution to Locke's solution caused a transient secretion (see Fig. 3), for clarity 'spontaneous' outputs have been omitted from (*c*).

*Effect of ACh in Na-deficient Locke's solution*

Similar experiments were done to test the effect of ACh during perfusion with Na-deficient Locke's solution in which NaCl was replaced with an isosmotically equivalent amount of sucrose. The solution was otherwise identical with the control Locke's solution and like it contained a small amount of sodium in the form of phosphate buffer. In these experiments a large output of catecholamines was still evoked by ACh after 16 min perfusion with the Na-deficient medium (Fig. 2*b*).

When several such periods of Na deprivation were interpolated during prolonged perfusion with Locke's solution, the outputs of catecholamines in response to stimulation by ACh were strikingly diminished, not only when ACh was administered in the Na-free Locke's solution but also when it was given in Locke's solution. In this respect the experiments differed from those involving K-free perfusion, where the deterioration was not greatly in excess of that which occurred in glands perfused throughout with Locke's solution. It was also noted that unlike perfusion with K-free Locke's solution, perfusion with a reduced concentration of sodium caused a progressive increase in the 'spontaneous' output of catecholamines.

*Effect of ACh in Ca-free Locke's solution*

The omission of calcium from the perfusion fluid had an effect strikingly different from the effect of omitting sodium or potassium. Perfusion with Ca-free Locke's solution for 16 min reduced the amount of catecholamines released by ACh to a very small fraction (5 % to less than 1 % in different tests) of the control value obtained in Locke's solution (Fig. 2*c*). The effect was reversible and could be repeatedly obtained in a single preparation.

During the periods of perfusion with Ca-free Locke's solution the spontaneous output of catecholamines tended to fall, and the total loss of catecholamines from the gland during such a period, before the introduction of ACh in Ca-free Locke's solution, was a small fraction (less than 5 %) of the outputs in response to ACh given earlier or later in the experiment while perfusing with Locke's solution. Clearly there was no significant depletion of catecholamines at the time when ACh, in Ca-free Locke's solution, failed to elicit secretion.

*Release of catecholamines by calcium*

It was found that the effect of calcium deprivation was not confined to suppression of the response to ACh. After a period of perfusion with Ca-free Locke's solution, some change occurred in the gland such that large amounts of catecholamines were released merely by reintroducing Locke's solution containing the normal amount of calcium (Fig. 3*a*). It does not

follow from such an experiment, however, that this effect of reintroducing calcium is due to an action on the adrenal medullary cells directly. The effect could be on the splanchnic nerve endings, since Hutter & Kostial (1955) found during experiments on the superior cervical ganglion perfused with Locke's solution that the amount of ACh released by nerve stimulation was occasionally enhanced if the test were preceded by a period of perfusion with Ca-free Locke's solution. If in our experiments there

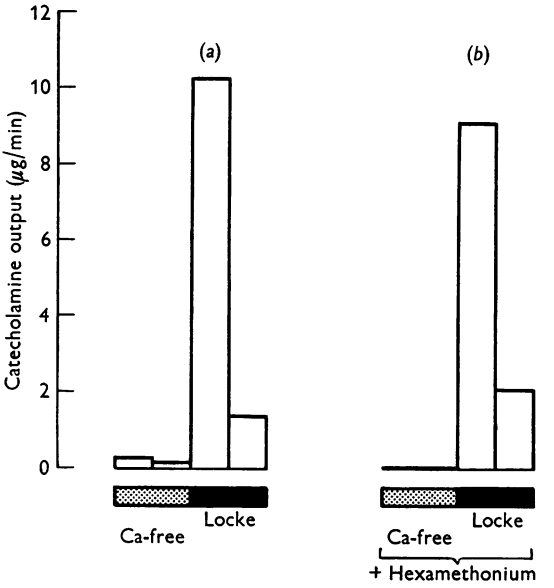


Fig. 3. Release of catecholamines by Locke's solution (indicated by solid horizontal bar) following perfusion with Ca-free Locke's solution (indicated by stippled horizontal bar). (*a*), both adrenals perfused *in situ*, (*b*), left adrenal perfused *in vitro*: hexamethonium ($5 \times 10^{-4}$ g/ml.) present throughout this experiment.

were any random activity in the sympathetic nerves, such as injury discharge, then reintroduction of calcium might increase the amount of ACh released and thereby increase catecholamine output. It is highly improbable, however, that a presumably small and random effect could account for a release of catecholamines of the magnitude found in our experiments. The amounts of catecholamines released by reintroduction of Locke's solution were usually several times greater than those we were able to release by maximal stimulation of the splanchnic nerves in Locke's solution. The possibility of such an indirect mechanism of catecholamine release was excluded by experiments with hexamethonium. This substance is known to suppress the secretory response to splanchnic nerve stimulation (Paton & Zaimis, 1952), and we have found that under our conditions

hexamethonium ($5 \times 10^{-4}$ g/ml.) reduces the response to maximal splanchnic nerve stimulation from about 2·5 $\mu$g/min to about a tenth of this value (this effect is illustrated in Fig. 5). As is seen from Fig. 3*b*, hexamethonium in this concentration did not prevent the reintroduction of calcium from causing its customary powerful effect and raising secretion to about 9 $\mu$g/min.

The finding that calcium in a normal concentration could stimulate adrenal medullary secretion in these abnormal conditions prompted us to test whether *excess* calcium would stimulate the gland during perfusion with Locke's solution. When perfusion was switched from Locke's solution to Locke's solution plus calcium 20 mM (i.e. to a total of 22·2 mM Ca) there was no obvious increase in secretion.

*Quantitative relation between secretion and calcium concentration*

It was found that there was a quantitative relation between the amount of catecholamines released by ACh and the concentration of calcium in the perfusion fluid over a wide range. This was shown by experiments in which responses to ACh ($10^{-5}$ g/ml.) were tested alternately in Locke's solution and in a solution otherwise identical but containing more, or less, calcium. One of eight such experiments is illustrated in Fig. 4, which shows that catecholamine output was more than doubled when the calcium concentration was raised to 17·6 mM. This was the largest effect observed: higher concentrations of calcium were not studied. With 8·8 mM Ca the response to ACh was about 50 % greater than the control value, while with 0·5 mM Ca it was about half the control value.

*The effect of Ca lack on the response to potassium*

It is not improbable that ACh may cause catecholamine secretion by depolarizing adrenal medullary cells, for it depolarizes the homologous sympathetic ganglion cells (Paton & Perry, 1953). Moreover, excess potassium (which may be assumed to depolarize) is known to release catecholamines from the medulla. It was therefore of interest to see whether the response to potassium was also dependent on the presence of calcium. The addition of potassium (50 mM) to Locke's solution caused the gland to secrete at a rate of 10 $\mu$g/min or more. This same concentration of potassium, however, was almost without effect when added to Ca-free Locke's solution at the end of 16 min perfusion with this medium. Although it is known that potassium excites adrenal medullary cells directly (Vogt, 1952), experiments were done with hexamethonium to rule out the possibility that the effect of potassium observed in our experiments was indirect and the consequence of stimulation of splanchnic nerve endings. One such

experiment is illustrated in Fig. 5, which shows that potassium evoked a powerful secretion under conditions in which the response to maximal splanchnic stimulation was enfeebled by hexamethonium, and also that potassium failed to release catecholamines when calcium was absent.

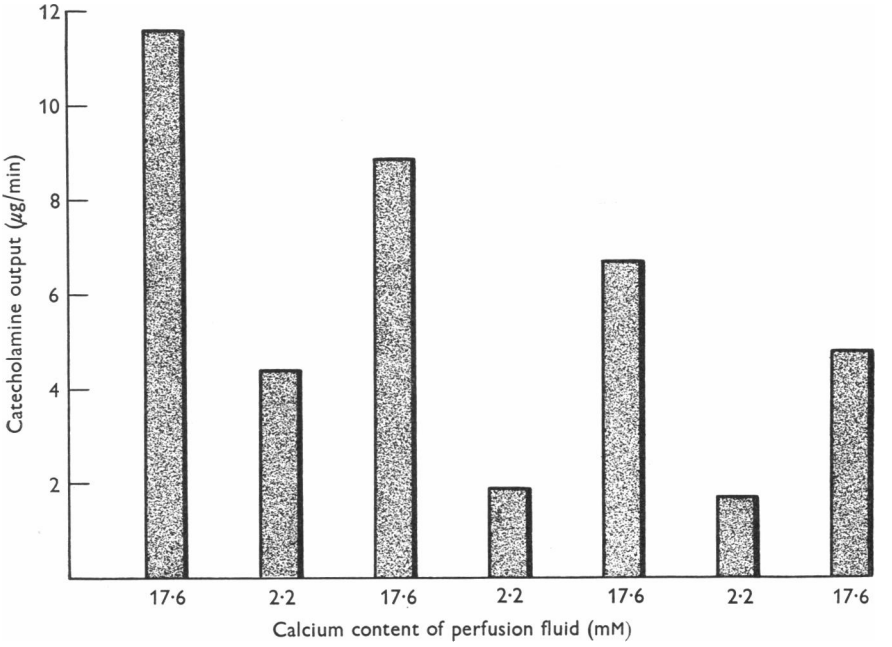


Fig. 4. The potentiating effect of excess calcium on the amount of catecholamine released by ACh. An adrenal gland was perfused *in vitro* alternately for 16 min periods with Locke's solution (2·2 mM Ca) and a 'high-calcium' Locke's solution (17·6 mM Ca). During the last 4 min of each period of perfusion with one or other solution, ACh $10^{-5}$ g/ml. was introduced for 30 sec. The vertical bars show the secretory responses obtained sequentially in this way.

### *Perfusion with sucrose plus calcium*

Some experiments were done to see whether calcium alone, in the absence of sodium or potassium, might be sufficient to allow ACh to release catecholamines. Perfusion was carried out with isotonic sucrose containing calcium 2·2 mM and a small amount of sodium phosphate buffer (1·3 mM). A complication arose in that this Ca-sucrose solution itself caused a steadily mounting output of catecholamines reaching a level of several micrograms per minute. In such experiments (Fig. 6*a*) ACh failed to release further amounts of catecholamines, although the rate of secretion was usually well below that which might have been expected from ACh or the rate that could be achieved in different circumstances (e.g. Fig. 6*c*).

The strongly stimulant effect of the Ca-sucrose medium could not be attributed to the pH, for although acidity is known to cause release of catecholamines (von Euler & Stjärne, 1955) the solution was buffered to the same pH as the Locke's solution. Apparently the effect resulted from the withdrawal of sodium and potassium together. As we have described earlier, removal of one or other of these ions individually had comparatively minor effects on spontaneous release.

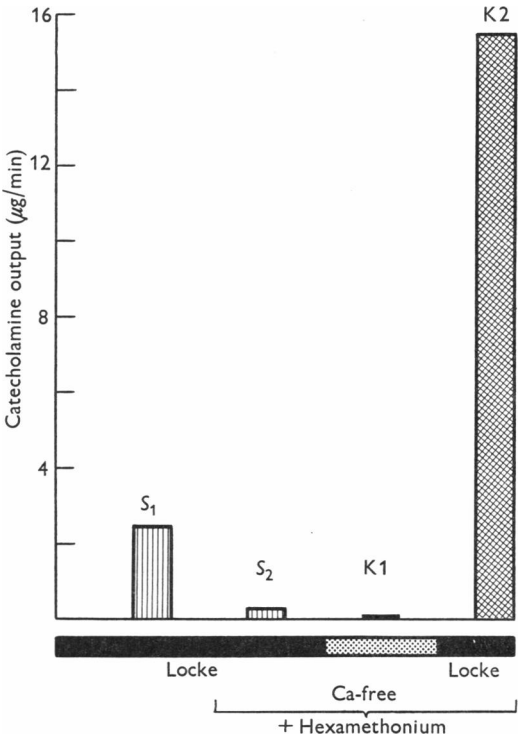


Fig. 5. An experiment on an adrenal gland perfused *in vivo* showing that potassium evokes medullary secretion in the presence of hexamethonium sufficient to suppress the stimulant effect of splanchnic nerve stimulation and that this effect depends on calcium.

The four responses were obtained sequentially at intervals of about 20 min. *S* 1 and *S* 2 show the catecholamine outputs to 4 min periods of maximal stimulation of the splanchnic nerve at 10 shocks/sec before and after the addition of hexamethonium $5 \times 10^{-4}$ g/ml. to the perfusion fluid (Locke's solution). K 1 and K 2 show the catecholamine outputs to 4 min periods of perfusion with potassium (56 mM) in Ca-free Locke's solution and Locke's solution, each containing hexamethonium $5 \times 10^{-4}$ g/ml. (Tonicity of the 'high-potassium' solutions was maintained by appropriate reduction of sodium.)

*Perfusion with pure isosmotic sucrose*

Perfusion with pure isosmotic sucrose free from calcium was also found to cause catecholamine release. This effect was seen not only when this medium was introduced after Locke's solution and when some calcium would still be present, but also in experiments in which Ca-free sucrose was given after a period of 16 min perfusion with Ca-free Locke's solution

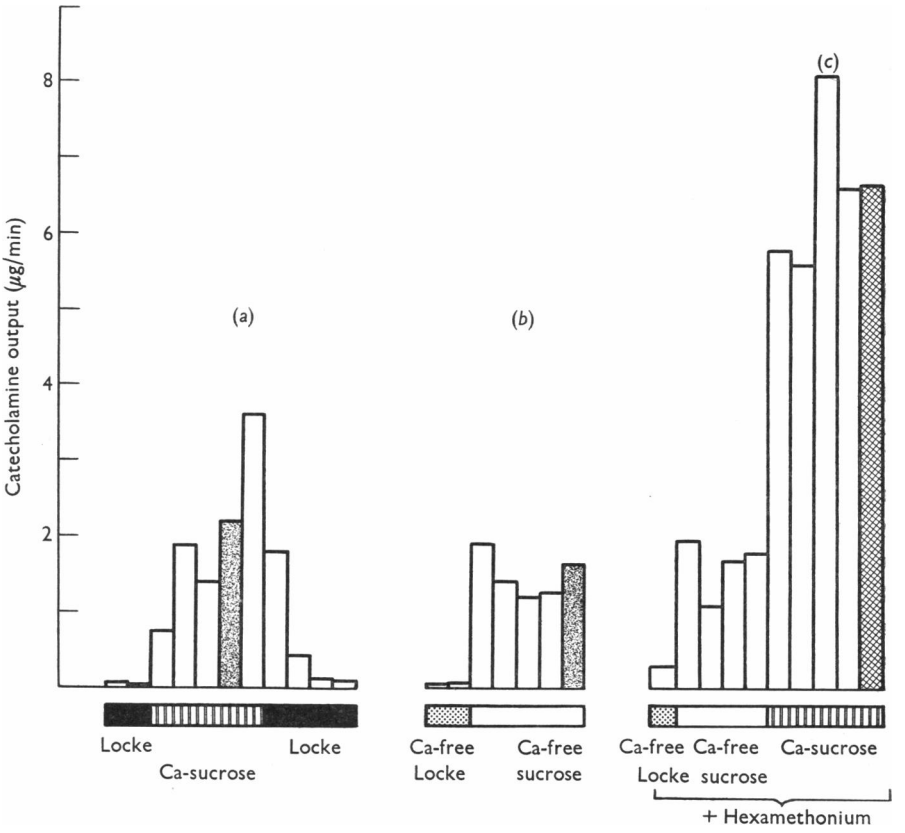


Fig. 6. Experiments on three adrenal glands perfused *in vitro* showing stimulation of catecholamine output by isosmotic sucrose with or without calcium, and in the presence or absence of hexamethonium. Catecholamine outputs in successive 4 min collection periods are shown. (*a*), the effect of sucrose plus 2·2 mM calcium following Locke's solution. (*b*), the effect of Ca-free sucrose following Ca-free Locke's solution. (*c*), the effect of adding calcium to Ca-free sucrose (hexamethonium $5 \times 10^{-4}$ g/ml. present throughout *c*).

ACh $10^{-5}$ g/ml. was present during the periods indicated by the solid vertical bars in (*a*) and (*b*). K (56 mM) was present during the period indicated by the cross-hatched vertical bar in (*c*) (isotonicity was maintained by appropriate reduction of sucrose).

(Fig. 6*b*). Such effects of Ca-free sucrose were also seen in glands perfused with hexamethonium (Fig. 6*c*) and must therefore involve the medullary cells directly. When calcium was added during such perfusion the output of catecholamines increased greatly, but it was not further increased by the addition of potassium (56 mM) to the sucrose medium (Fig. 6*c*).

## DISCUSSION

Acetylcholine clearly evokes adrenal medullary secretion through some calcium-dependent process. This seems to involve the release or ejection of catecholamines rather than their synthesis or storage, for there is no significant loss of catecholamines from the gland during Ca-free perfusion when ACh fails to cause secretion, and large amounts of catecholamines can be released in such conditions by other stimuli such as isosmotic sucrose.

It is known that calcium has an important action in maintaining cell membranes, in particular their impermeability to various ions (Frankenhaeuser & Hodgkin, 1957; Harris, 1960; Maizels, 1960) and a passive role of this sort might explain *some* of our results, such as the reduced efficacy of ACh when calcium is low and its lack of effect when calcium is absent. It offers, however, no ready explanation for the findings that the response to ACh is potentiated by calcium in high concentrations and that calcium itself may cause secretion. These suggest to us that calcium may play a more active role in the stimulant action of ACh. A clue to this role is provided by the evidence that calcium is only effective in releasing catecholamines after a period of calcium deprivation, and that as much as ten times the normal concentration of calcium fails to evoke secretion when added during perfusion with Locke's solution containing the normal amount of calcium. The stimulant effect of reintroducing calcium thus occurs in circumstances known to increase membrane permeability. The effect could thus be due to calcium ions penetrating the medullary cells. If the level of ionized calcium within the medullary cells is as low as it appears to be in various other cells (Keynes & Lewis, 1956; Harris, 1957), then an inward flux of calcium ions is to be expected if permeability to them is increased.

The possibility thus arises that ACh may cause secretion by acting on the membranes of the adrenal medullary cells to cause them to take up calcium ions. This does not seem unlikely. At each of the sites of cholinergic transmission where the role of ACh has been rigorously examined (e.g. the motor end-plate, del Castillo & Katz, 1956) it appears to be confined to the membrane, probably its outer surface, and to involve the production of some increase in membrane permeability to common species of ions. There is, moreover, evidence that ACh does not act directly on the intracellular

stores of catecholamines (the adrenal medullary granules) to cause release of catecholamines (Blaschko, Hagen & Welch, 1955; Schümann & Weigmann, 1960). If ACh acts on medullary cells to increase their permeability to calcium, an inward movement of calcium ions would be expected for the reasons we have just given. Such an influx of calcium ions in response to ACh has already been found in muscles (Robertson, 1960; Shanes, 1961). It would also be expected that the amount of calcium ions passing inward would depend on the extracellular calcium-ion concentration, as has again been found in muscle (Holland & Sekul, 1959). Such an active role of calcium in the secretory response of the adrenal medulla to ACh would account not only for the depressant effects of low and zero calcium concentrations, but also for the potentiating effects of calcium concentrations above the normal. It would also be consistent with the finding that omission of potassium enhances the secretory response to ACh, for calcium influx into muscles is known to be enhanced in such circumstances (Niedergerke & Harris, 1957; Holland & Sekul, 1959).

The fact that isosmotic sucrose liberates catecholamines in the absence of extracellular calcium does not argue against the hypothesis that calcium links the ACh stimulus with the secretory response, but indicates that catecholamines may also be released by other mechanisms. Whether it is the sucrose, or the lack of sodium and potassium, which is responsible for the release is uncertain, but the latter possibility is suggested by the fact that sucrose containing potassium 5·6 mM was much less effective. It is known that in pure sucrose the catecholamine granules isolated from the adrenal medullary cells tend to lose their amine content (Hillarp, 1958; Schümann & Weigmann, 1960), but this effect is rather small at the temperatures at which we worked, and moreover it seems improbable that significant quantities of sucrose penetrate the medullary cells. The effect of isosmotic sucrose on the whole gland seems most probably to be a membrane phenomenon. It is of interest that the almost complete replacement of sodium with sucrose, which we found causes a progressive increase in the 'resting' discharge of catecholamines, causes an increased uptake of calcium and its accumulation in heart muscles (Niedergerke & Harris, 1957).

Although calcium is obviously able to evoke catecholamine secretion in the whole gland, Hillarp (1958) has reported that the addition of calcium 1 mM does not increase the release of catecholamines from isolated adrenal medullary granules suspended in sucrose. Although this might simply mean that some other intracellular constituent is required along with calcium, there are grounds for believing that the process of secretion occurring in the whole cell does not involve the intracellular disruption of these granules or indeed the leakage of catecholamines from them. Rather, from electron-microscopic studies it seems that the process of adrenal medullary secretion involves a complex chain of events (referred to as 'vesiculation and membrane flow') during which the secretory granules are first attached to the membrane and their contents then extruded through it (De Robertis, Nowinski & Saez, 1960). This may be the process

which is calcium-dependent. Calcium is known to have an influence not only on cell membranes but also on the physico-chemical properties of cell sap (Heilbrunn, 1943, 1956; Hodgkin & Katz, 1949; Chambers & Kao, 1952).

It is not known if ACh depolarizes adrenal medullary cells but it acts in this way at other sites including the developmentally homologous ganglion cells (Paton & Perry, 1953). Since we have found that potassium in concentrations which presumably cause considerable depolarization also excites medullary secretion through some calcium-dependent process, and since depolarization is known to cause an influx in calcium ions in muscles and nerves (Bianchi & Shanes, 1959; Hodkgin & Keynes, 1957) it is not improbable that ACh could cause calcium influx at the medulla by depolarization. However, the omission of sodium from the perfusion fluid for 16 min did not seriously depress the response to ACh although it might be expected to impair depolarization due to sodium influx, and it is thus possible that ACh may stimulate calcium uptake without depolarization. (The loss of the stimulant response to ACh after more lengthy perfusion with sodium-free Locke's solution may be due to desensitization of ACh receptors such as appears to occur at motor end plates in such a medium (del Castillo & Katz, 1955).

Because of the common developmental origins of the adrenal medullary cells and the post-ganglionic sympathetic neurones, the present experiments obviously provide grounds for supposing that calcium may be similarly involved in the secretion of noradrenaline on the arrival of impulses at sympathetic nerve endings. In nerves which are cholinergic calcium appears to act in this way as a link between the nerve impulse and the secretion of ACh, and calcium penetration into the nerve ending during activity has been postulated as the trigger for ACh secretion (Birks & MacIntosh, 1957; Hodgkin & Keynes, 1957). Although the stimulus to secretion in nerves is the action potential while that to the medullary cell is ACh, a common mechanism—depolarization—may underly both responses. A further inference from our experiments is that other biologically active substances may be released from their intracellular stores by calcium entry. Calcium is required for the release of histamine and 5-hydroxytryptamine during the antigen–antibody reaction (Humphrey & Jaques, 1955) and the amount of histamine released varies with the calcium-ion concentration (Mongar & Schild, 1958).

Finally, each of the main pieces of evidence which together suggest to us that calcium somehow links the ACh stimulus to the secretory response in the adrenal medulla has a parallel in the evidence which has led others to propose that calcium influx links stimulation with muscular contraction (Heilbrunn, 1943, 1956; Sandow, 1952; Shanes, 1958). Thus, omission of

calcium leads to failure of contraction (Frank, 1960): force of contraction varies with extracellular calcium concentration (Niedergerke, 1956): calcium causes contraction provided the membrane barrier to it is removed (Podolsky & Hubert, 1961): and omission of potassium increases force of contraction (Niedergerke & Harris, 1957). There are obvious similarities between the process which Sandow has described as 'excitation-contraction coupling' and what we may term 'stimulus-secretion coupling'. The extent of the similarity and its significance remains to be discovered.

SUMMARY

1. Experiments have been made to determine how ACh, the chemical transmitter of sympathetic effects at the adrenal medulla, causes the medullary cells to secrete.

2. In perfused cat's adrenals a profound lowering of the sodium content of the perfusion medium for 16 min did not cause much reduction in the output of catecholamines in response to ACh. When potassium was omitted for a similar time the response to ACh was increased.

3. The omission of calcium, however, virtually abolished the secretory response of the adrenal medulla to ACh. The effect did not seem attributable to reduction of catecholamine stores and was considered to involve the process of catecholamine release.

4. Excess potassium (56 mM) also failed to have its usual powerful stimulant effect on catecholamine secretion when given during perfusion with Ca-free Locke's solution.

5. A quantitative relation was found between the amount of catecholamines released by ACh and the concentration of calcium in the perfusion fluid at values above as well as below the normal 2·2 mM Ca.

6. Calcium itself (2·2 mM) caused a vigorous discharge of catecholamines when introduced after a 16 min period of perfusion with Ca-free Locke's solution.

7. Isosmotic sucrose evoked release of catecholamines; addition of calcium greatly augmented this effect.

8. It is suggested that the role of ACh as a transmitter at the adrenal medulla is to cause some brief change in medullary cells which allows calcium ions to penetrate them and trigger the catecholamine ejection process.

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